

APPLICATION
FOR
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TITLE: GUIDED DEVELOPMENT AND SUPPORT OF
HYDROGEL-CELL COMPOSITIONS

APPLICANT: CHARLES A. VACANTI, JOSEPH P. VACANTI AND
MARTIN P. VACANTI

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GUIDED DEVELOPMENT AND SUPPORT OF HYDROGEL-CELL COMPOSITIONSCross-Reference to Related Application

This application is a continuation-in-part of U.S.
5 Serial No. 09/066,038, filed on April 24, 1998, which is
incorporated by reference herein in its entirety.

Field of the Invention

The invention relates to methods of generating
tissue such as cartilage, bone, nervous system, skin,
10 organs, and epithelial layers, to repair or replace tissue
damaged by disease or injury, augment existing tissue, or
create tissue not originally present.

Background of the Invention

There is much interest within the medical community
15 for methods and materials for use in reconstructive surgery
of tissue. An emerging approach is tissue engineering, in
which new tissue is grown from tissue-precursor cells and
engineered into desired shapes and structures. The types of
tissues that can be grown and engineered include, for
20 example, bone, and cartilage.

One of the primary uses for replacement cartilage is
to correct defects in the articular surface of various
joints. For example, a damaged cartilage meniscus in a
patient's knee can be replaced with an artificially
25 engineered meniscus. See, for example, U.S. Patent No.
5,041,138 (Vacanti et al.). In other examples of tissue
engineering, hollow spaces or lumens within tissues can be
filled with tissue-precursor cells suspended in, for
example, gelatin, collagen, fibrin, or various hydrogels.
30 See, for example, International Patent Application WO
94/25080 entitled "Injectable polysaccharide-cell
compositions" (Griffith-Cima et al.).

Summary of the Invention

The invention features a method of generating new tissue in a patient by delivering a liquid hydrogel-cell composition, which contains a hydrogel and tissue precursor cells, into a permeable, biocompatible support structure. The hydrogel-cell composition fills the permeable support structure and takes the shape of the support structure as the composition solidifies and new tissue is formed as the cells grow and multiply. After being saturated with the hydrogel-cell composition, the support structure can be implanted into a patient. Alternatively, the support structure can be filled with the liquid hydrogel-cell composition after it is implanted into the patient. For example, the support structure can be implanted and subsequently injected with the hydrogel-cell composition using a syringe or catheter. The support structure is shaped to correspond to the desired tissue to be grown, e.g., a bone, a meniscus for a knee, an ear, an organ, a nose, a portion of the spinal cord, or other tissue, e.g., cartilaginous tissue or autonomic nervous system tissue.

The combination of suspending the tissue precursor cells in the hydrogel and delivering the hydrogel-cell composition into a support structure profoundly improves the quality of the new tissue growth and the range of tissue shapes and structures that can be grown. The hydrogel-cell composition forms a uniform distribution of cells with a well-defined and precisely controllable density. Moreover, the hydrogel can support very large densities of cells, e.g., 50 million cells/ml. These factors improve the quality and strength of the new tissue. In addition, the hydrogel allows diffusion of nutrients and waste products to, and away from, the cells, which promotes tissue growth.

The support structure provides support for the hydrogel-cell composition and guides the development and shape of the growing tissue, which fills the support structure. Since it is the hydrogel that sustains and
5 suspends the tissue precursor cells, rather than the support structure, the support structure can have any shape or structure from a wide range of shapes and structures. In particular, suitable support structures are sufficiently permeable to carry the hydrogel-cell composition and allow
10 the exchange of nutrients and waste products between the hydrogel within the support structure and bodily fluids outside the support structure. Furthermore, the support structures can be biodegradable or biocompatible. Examples of support structures include sponges, foams, corals, rigid
15 inorganic, ceramic, or metal structures having internal pores such a honeycomb structure made from titanium, a skeleton of fine struts, such as a mesh of thin interwoven polymer fibers, and a skeleton of thick struts, such as a network of metal, inorganic, ceramic, or plastic rods.

20 In general, in one aspect, the invention features a method for generating new tissue, the method including: obtaining a liquid hydrogel-cell composition including a hydrogel and tissue precursor cells; delivering the liquid hydrogel-cell composition into a permeable, biocompatible
25 support structure; and allowing the liquid hydrogel-cell composition to solidify within the support structure and the tissue precursor cells to grow and generate new tissue.

The liquid hydrogel-cell composition can be delivered to the support structure by injecting the liquid
30 composition into the support structure.

The method can further include implanting the support structure into an animal, e.g., a mammal such as a human. After the support structure is implanted into the

animal, the hydrogel-cell composition can be delivered to the support structure.

In another aspect, the invention features a tissue forming structure that includes: a permeable, biocompatible support structure having a predetermined shape that corresponds to the shape of desired tissue; and a hydrogel-cell composition at least partially filling the support structure, wherein the hydrogel-cell composition includes a hydrogel and tissue precursor cells.

For example, the cells can be bone forming cells and the support structure can include porous hydroxyapatite. Also, the hydrogel-cell composition can be a solidified suspension of hydrogel supporting dispersed tissue precursor cells.

The methods and tissue forming structure can include any of the following features.

The support structure can include any of ceramic material, metal, sponge, foam, porous hydroxyapatite, a mesh of fibers (e.g., a mesh of polyglycolic acid fibers and polylactic acid), and various polymers. For example, the support structure can be formed from polyanhydride, polyorthoester, polyglycolic acid, polylactic acid, polyglactin, or combinations thereof. Also, the support structure can be compressible or rigid, or elastic.

The support structure can be shaped in the form of desired tissue, such as in the form of articular cartilage adjacent a joint, a bone, a portion of a bone, a bone defect, or a brain or other central nervous system tissue defect. The support structure can be also shaped in the form of a cylinder having the diameter of the spinal cord of a mammal to be treated. The support structure can also be biodegradable.

The hydrogel can include any of the following:
polysaccharides, proteins, polyphosphazenes,
poly(oxyethylene)-poly(oxypropylene) block polymers,
poly(oxyethylene)-poly(oxypropylene) block polymers of
5 ethylene diamine, poly(acrylic acids), poly(methacrylic
acids), copolymers of acrylic acid and methacrylic acid,
poly(vinyl acetate), and sulfonated polymers.

The tissue precursor cells can include any of the
following: epidermal cells, chondrocytes and other cells
10 that form cartilage, macrophages, dermal cells, muscle
cells, hair follicles, fibroblasts, organ cells, osteoblasts
and other cells that form bone, endothelial cells, mucosal
cells, pleural cells, ear canal cells, tympanic membrane
cells, peritoneal cells, Schwann cells, corneal epithelial
15 cells, gingiva cells, neural cells, neural stem cells such
as central nervous system (CNS) stem cells, e.g., spinal
cord or brain stem cells, as well as autonomic nervous
system (ANS) stem cells, e.g., post-ganglionic stem cells
from the small intestine, bladder, liver, lung, and heart,
20 (for engineering sympathetic or parasympathetic nerves and
ganglia), tracheal epithelial cells, hepatocytes, pancreatic
cells, and cardiac cells. The tissue precursor cells can
also be neuroendocrine stem cells.

In another aspect, the invention features isolated,
25 mammalian adult autonomic nervous system neural stem cell.
These stem cells can be isolated from any innervated tissues
in the body, including the heart, bladder, intestine, lung,
liver, and kidney tissue. The invention also includes
isolated, mammalian neuroendocrine stem cells, e.g., adult
30 cells, such as those isolated from the pancreas (adult or
fetal), or adult cells isolated from the adrenal medulla.

The invention also features, in another aspect, a
method of treating defective nervous tissue by locating the

physical boundaries of the defective tissue; removing the defective tissue to create a cavity and exposing healthy nervous tissue at the surfaces of the cavity; loading a hydrogel-neural stem cell composition into a support structure in the general size and shape of the cavity, wherein the neural stem cells are selected to differentiate into the healthy nervous tissue; and implanting the support structure into the cavity, thereby treating the defective nervous tissue. The defective nervous tissue can be CNS tissue, e.g., in the brain or spinal cord, ANS tissue, or neuroendocrine tissue. The neural stem cells can be isolated from the healthy nervous tissue. In this method, a spacer can be implanted into the cavity temporarily, and then replaced with the support structure.

The hydrogel-neural stem cell composition can be loaded into the support structure before or after the structure is implanted into the cavity.

In another aspect, the invention features a method of isolating and culturing undifferentiated, mammalian neural stem cells, e.g., adult cells, such as neuroendocrine cells or autonomic nervous system cells, by obtaining a sample of nervous system tissue (and keeping the tissue at about 40°F or less); dissociating the tissue in a first liquid; separating solid matter from the first liquid; mixing the solid matter into a second liquid and degrading tissue debris (e.g., with an enzyme such as trypsin) to form a mixture; triturating the mixture to separate cells and break differentiated cells; separating solids from the triturated mixture and suspending the solids in a culture medium including epidermal growth factor (EGF) and optionally fibroblast growth factor; culturing the culture medium and passaging surviving cells at least every fourth day, wherein surviving cells are neural stem cells and are

triturerated and resuspended in fresh culture medium at each passage; and repeating the culturing and passaging steps to maintain the culture of isolated, undifferentiated neural stem cells.

5 A "hydrogel" is a substance formed when an organic polymer (natural or synthetic) is set or solidified to create a three-dimensional open-lattice structure that entraps molecules of water or other solution to form a gel. The solidification can occur, e.g., by aggregation,
10 coagulation, hydrophobic interactions, or cross-linking. The hydrogels employed in this invention rapidly solidify to keep the cells at the application site, thereby eliminating problems of phagocytosis or cellular death and enhancing new cell growth at the application site. The hydrogels are also
15 biocompatible, e.g., not toxic, to cells suspended in the hydrogel.

 A "hydrogel-cell composition" is a suspension of a hydrogel containing desired tissue precursor cells. These cells can be isolated directly from a tissue source or can
20 be obtained from a cell culture. A "tissue" is a collection or aggregation of particular cells embedded within its natural matrix, wherein the natural matrix is produced by the particular living cells.

 "Tissue precursor cells" are cells that form the
25 basis of new tissue. Tissue cells can be "organ cells," which include hepatocytes, islet cells, cells of intestinal origin, cells derived from the kidney, cells of hair follicles, cells from the vitreous humor in the eyes, cells from the brain, and other cells acting primarily to
30 synthesize and secret, or to metabolize materials. Tissue precursor cells include so-called "stem" cells (or "progenitor" cells) that are undifferentiated precursor cells that can form a number of different types of specific

cells under different ambient conditions. For example, spinal cord stem cells (or progenitor cells) are undifferentiated nerve cells that can be induced to form various differentiated cell types in mature spinal cord tissue such as motor neurons, sensory neurons, internuncial neurons, preganglionic sympathetic and parasympathetic neurons, and peripheral nervous system cells.

One method of inducing maturation of these cells is by altering the physical properties of the support scaffold. Contact alone with these scaffolds can cause the cells to differentiate. Also, addition of growth factors or other hormones into the hydrogel can drive differentiation of these cells.

An "isolated" tissue precursor cell, such as an isolated neuroendocrine stem cell or ANS stem cell, is a cell that has been removed from its natural environment in a tissue within an animal, and cultured *in vitro*, at least temporarily. The term covers single isolated cells, as well as cultures of "isolated" stem cells, that have been significantly enriched for the stem cells with few or no differentiated cells.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, useful methods and materials are described below. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In case of conflicting subject matter, the present specification, including definitions, will control.

In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

The invention has many advantages. For example, upon gelling, the hydrogel-cell composition is supported by the support structure and forms a uniform suspension in which nutrients can readily diffuse to the cells and waste products can diffuse away from the cells. As a result, the hydrogel keeps the cells viable and allows vascularization of the suspension, ultimately resulting in the growth of new tissue and its engraftment to the patient's body. The permeable support structure, into which the liquid hydrogel-cell composition is delivered, provides a shape and structure for the solidifying hydrogel-cell composition while still allowing nutrients and waste products to diffuse to and from the cells within the hydrogel. Thus, the support structure guides the development and shape of the new tissue, and does so with the ability to resist external stresses from the environment and surrounding tissues, i.e., the support structure is immediately weight-bearing, for example if the structure is used to replace a bone or cartilage. Moreover, the weight bearing characteristic of the support structure in bone or cartilage enables stresses to be transmitted across the hydrogel-cell suspension that will encourage bone or cartilage development in the hydrogel-cell suspension.

In the absence of such a support structure, a patient would not be able to apply any weight or stress to the hydrogel-cell composition because such forces would cause the hydrogel-cell composition to become distorted and displaced. This is similar with any replacement of structural tissues which are exposed to stress, such as ligaments, tendons, and bones anywhere within the body. Note also that the support structure accommodates not only

compressive forces but also tensile forces, such as when one pulls on a tendon or stretches skin. In such cases, the support structure mimics the elasticity of the tendon or skin, and has ends that are typically sutured or adhered to adjacent tissue.

Composite support structures are also possible. For example, one can create a support structure that has a hollow tube of solid material, e.g., coral, on the outside loaded with a hydrogel-osteoblast (or other bone precursor cells) composition, and a softer support in the center such as a fiber mesh loaded with a hydrogel-spinal cord stem cell composition.

Also, the support structure maintains the structural integrity and the desired shape of the hydrogel-cell composition without altering the physical characteristics of the hydrogel-cell composition in ways that can harm the cells and limit the diffusion of nutrients and waste products to and from the cells.

Furthermore, the support structure can be designed to be compressible and resilient so that it can be easily implanted into a patient through, for example, a cannula, endoscope, or arthroscope. Thus, the support structure can be cut or molded into the shape of the desired tissue to be grown, implanted into the patient in a deformed state through a cannula, allowed to expand within the desired body cavity, and subsequently injected with the liquid hydrogel-cell composition. The subsequent growth of new tissue will take the shape of the support structure.

The hydrogel can also be designed to gradually expand with time to fill cavities, or encourage production of a larger mass of tissue with time. For example, in breast and liver, slow expansion will solve the problem of creating bulk by enabling nutrition of the individual cells

as the tissue grows. As another example, the hydrogels can be used within the backbone to treat portions of the spinal cord damaged by disease that leaves the vertebrae intact.

5 In addition, the hydrogel-cell compositions, especially compositions of neural stem cells or muscle cells, can be loaded onto support structures that can conduct electrical current that can be used to promote cell growth and control direction of cell growth and migration.

10 Other features and advantages of the invention will be apparent from the following detailed description, and from the claims.

Brief Description of the Drawings

Fig. 1 is a schematic of a permeable support structure filled with a hydrogel-cell composition.

15 Fig. 2 is a photograph of a permeable, polymeric support structure in the shape of a rat femur.

Fig. 3 is a photograph of a nude mouse in which the support structure of Fig. 2 has been implanted subcutaneously.

20 Fig. 4 is a photograph of the support structure of Fig. 2 after it has been injected with a hydrogel-cell composition containing periosteal cells and subsequently removed from the mouse shown in Fig. 3.

25 Fig. 5 is a photograph showing the histology of the support structure of Fig. 4.

Detailed Description

The invention provides methods and compositions for growing new tissue such as, for example, cartilage, bone, skin, epithelial layers, new organs, and central nervous
30 system tissue including brain tissue, spinal cord tissue, and peripheral and autonomic nerves, by using a hydrogel-

cell composition. To guide the development and shape of the new tissue, the hydrogel-cell composition is delivered into a permeable support structure, which is implanted into a patient either before or after the hydrogel-cell composition is delivered to the support structure. In the following subsections, suitable support structures, hydrogels, cells such as precursor or stem cells, and delivery methods will be described, along with illustrative examples.

Support Structures

The support structure provides the shape and support for the solidifying hydrogel-cell composition. It is permeable, typically having pore-like cavities or interstices that are filled by the liquid-hydrogel composition. For example, the support structure can be a porous polymer mesh, a natural or synthetic sponge, a piece of coral or hydroxyapatite having a desired porosity, or a matrix of metallic, inorganic, ceramic, or plastic struts. The permeability of the support structure allows diffusion of nutrients outside the structure into the hydrogel-cell composition filling the support structure (i.e., where the cells are) and the diffusion of waste products produced by the cells out of the structure through the hydrogel-cell composition, thereby promoting growth of the cells in the hydrogel-cell composition. Although other liquids, e.g., body fluids, may occupy the support structure prior to being filled with the liquid hydrogel-cell composition, the hydrogel-cell composition will displace these liquids and rapidly harden within the support structure.

The support structure is shaped in the form of the tissue to be grown. For example, the support structure can be shaped as a piece of cartilaginous tissue, such as a meniscus for a knee or elbow, a piece of bone to repair a

bone defect, an ear, a nose, an internal organ, a ligament, a tendon, the trachea (as a conduit), mandibles, spinal cord, or a portion of the brain or skin. Depending on the material from which it is made, the support structure can be
5 shaped by cutting, molding, or casting, or any other method that produces the desired shape for the support structure.

The support structure is also biocompatible (e.g., not toxic to the cells suspending in the hydrogel) and, in some cases, the support structure can be biodegradable. The
10 support structure can be shaped either before or after the hydrogel-cell composition fills the support structure. For example, a partially flexible support structure can be filled with the hydrogel-cell composition and molded into a desired shape, e.g., by hand, as the hydrogel hardens.

15 In some cases, it is desirable that the support structure be flexible and/or compressible and resilient. In particular, in these cases, the support structure can be deformed as it is implanted, allowing implantation through a small opening in the patient or through a cannula or
20 instrument inserted into a small opening in the patient. After implantation, the support structure expands into its desired shape and orientation.

The hydrogel-cell composition can be injected into the support structure either before or after the support
25 structure is implanted into a patient. As the hydrogel solidifies, it will adopt the flexibility and resiliency of the support structure. Such resiliency and flexibility allows a support structure that is saturated with hydrogel-cell composition to accommodate compressive and tensile
30 forces. For example, a tissue replacement for a tendon, ligament, meniscus, or piece of skin can accommodate tensile forces associated with pulling and stretching the tissue replacement and can also accommodate compressive forces

associated with weight bearing by the tissue replacement. In addition, the flexibility and resiliency of the support structure can accommodate minimally invasive delivery of the tissue replacement, e.g., the support structure can be
5 compressed and pushed into the patient through a cannula, and subsequently allowed to expand once positioned within the patient.

The support structure can be formed of a biocompatible, or biodegradable, synthetic polymer such as a
10 polyanhydride, polyorthoester, or polyglycolic acid. The polymer should be chosen so that the resulting support structure provides adequate shape and support for the cell-containing hydrogel suspension and allows nutrients to diffuse to the cells and promote cell growth and
15 proliferation. Factors, including nutrients, growth factors, inducers of differentiation or dedifferentiation, products of secretions, immunomodulators, inhibitors of inflammation, regression factors, biologically active compounds which enhance cell differentiation or allow
20 ingrowth of the lymphatic network or nerve fibers, and drugs, can be incorporated into the polymer support structure.

An example of a suitable polymer is polyglactin, which is a 90:10 copolymer of glycolide and lactide and is
25 manufactured as VICRYL™ braided absorbable suture (Ethicon Co., Somerville, NJ).

Polymer fibers can be compressed together in a felt-like polymer mat, which is then cut or otherwise shaped into the desired support structure. Alternatively, the polymer
30 fibers can be compressed together in a mold that casts the compressed fibers into the desired shape for the support structure. In some cases, additional polymer can be added

to the polymer fibers as they are molded to impart additional structure to the fiber mesh.

For example, polyglycolic acid can be configured as a mesh of fibers which can be compressed into a porous mat, which is then cut or otherwise shaped into the desired shape and then immersed in a solution of polylactic acid (e.g., a 1.0 percent solution) to bond the fibers and allow them to retain their shape to form the support structure. The polylactic acid binds the crosslinks of the polyglycolic acid fibers and coats these individual fibers, which fixes the shape of the molded fibers. The polylactic acid also fills in the spaces between the fibers. Thus, the porosity of the support structure can be specified by introducing an appropriate amount of polylactic acid. The pressures required to mold the fiber mesh into the desired shape can be quite moderate. All that is required is that the fibers are held in the desired shape for a time sufficient for the binding and coating action of polylactic acid. Suitable lengths and thicknesses of the fiber will depend on the desired shape and purpose of the support structure. For example, the strength of the fibers, which is proportional to their thicknesses, should be sufficient to resist the external forces that will be applied to that support structure.

Alternatively, or in addition, the support structure can include other types of polymer fibers, or polymer structures produced by techniques known in the art. For example, thin polymer films can be obtained by evaporating solvent from a polymer solution. These can be cast into a desired shaped if the polymer solution is evaporated from a mold having the relief pattern of the desired shape. Polymer gels can also be molded into thick, permeable

polymer structures using compression molding techniques known in the art.

5 The support structures can also be made from porous hydroxyapatite, e.g., made from chemically-altered marine coral (e.g., Interpore®), or hydroxyapatite paste which can be modified to increase the porosity and then molded into desired shapes. Unaltered marine coral can be used, but is resorbed by the body. The hydroxyapatite is machined into the shape of the desired support structure using
10 instrumentation known in the art, such as a Dremel drill. The use of hydroxyapatite or coral as the support structure is especially appropriate for growing tissue in bone defects. To deposit the hydrogel-cell composition into the coral a needle may be inserted into a pore, deep into the
15 coral, and the hydrogel-cell composition is injected as a liquid. Since pores of the coral are interconnected with one another, the liquid hydrogel-cell composition permeates through the coral and saturates the coral, providing an even distribution of cells.

20 Alternatively, the hydrogel can be pulled through a support structure by suction, or the support structure can be immersed in a hydrogel solution prior to cross-linking with an agent added to cross-link the hydrogel in situ within the support scaffolding. As another alternative,
25 some hydrogels can be cross-linked by photosensitization. This can be accomplished once the support scaffold is saturated with the hydrogel.

The size and shape of the support structure corresponds to the size and shape of the tissue to be grown, e.g., the size and shape of a cartilage replacement, a bone
30 defect, an ear, a nose, or a cavity created in a portion of defective nervous tissue. For example, a support structure for a human leg bone, which is porous in its center, can be

as long and thick as a human leg bone. In such a case, the hydrogel-cell composition fills both the outer layer of the support structure and the central porous area, or it can fill only the outer layer leaving the central porous area empty to serve as a canal for delivering nutrients up to the inner wall of the tissue developing at the outer layer.

In another example, a support structure used to repair a spinal cord injury is a cylinder having a dimension corresponding to the dimensions of the damaged spinal cord. The support structure gives not only structural cues, but also enhances cell differentiation after cells are implanted and migrate through the hydrogel to contact the support structure. Growth factors such as epidermal growth factor (EGF) and fibroblast growth factor (FGF) can be added to the hydrogel to enhance differentiation, multiplication, and to inhibit fibrosis.

Many other types of support structures are also possible. For example, the support structure can be formed from sponges, foams, corals, biocompatible inorganic, ceramic, or metal structures having internal pores such a honeycomb structure made from, e.g., titanium, a skeleton of biodegradable fine struts, such as a mesh of interwoven polymer fibers, and a skeleton of biocompatible thick struts, such as, for example, a network of metal, inorganic, ceramic, or plastic rods. These support structures are prepared using known methods.

Hydrogels

The hydrogels used to practice this invention should be biocompatible and biodegradable, preferably solidify rapidly in vivo, i.e., in about 5 minutes or less after being delivered to the implanted support structure, and should be capable of sustaining living cells.

The hydrogels used with neural stem cells are designed to be viscous enough to support the cells in three dimensions. If the suspension is not viscous enough, the cells will fall due to gravitational pull, will not be evenly distributed, and will change their phenotype to function in a less than optimal manner. The ideal is to maintain the cells suspended within the gel in a rounded configuration which is undifferentiated, until the hydrogel-cell composition is used to permeate a meshwork of fibers that support the hydrogel and enhance the stem cells to differentiate in their appropriate environment into the appropriate cell type. On the other hand, the hydrogel should not be too viscous to prevent all cell migration, or to inhibit cells from sending out dendrites and axons as they begin to differentiate after implantation.

Examples of different hydrogels suitable for practicing this invention, include, but are not limited to: (1) temperature dependent hydrogels that solidify or set at body temperature, e.g., PLURONICS™; (2) hydrogels cross-linked by ions, e.g., sodium alginate; (3) hydrogels set by exposure to either visible or ultraviolet light, e.g., polyethylene glycol polylactic acid copolymers with acrylate end groups; and (4) hydrogels that are set or solidified upon a change in pH, e.g., TETRONICS™.

Examples of materials that can be used to form these different hydrogels include polysaccharides such as alginate, polyphosphazenes, and polyacrylates, which are cross-linked ionically, or block copolymers such as PLURONICS™ (also known as POLOXAMERS™), which are poly(oxyethylene)-poly(oxypropylene) block polymers solidified by changes in temperature, or TETRONICS™ (also known as POLOXAMINES™), which are poly(oxyethylene)-

poly(oxypropylene) block polymers of ethylene diamine solidified by changes in pH.

Ionic Hydrogels

5 Ionic polysaccharides, such as alginates or
chitosan, can be used to suspend living cells. In one
example, the hydrogel is produced by cross-linking the
anionic salt of alginic acid, a carbohydrate polymer
isolated from seaweed, with ions, such as calcium cations.
The strength of the hydrogel increases with either
10 increasing concentrations of calcium ions or alginate. For
example, U.S. Patent No. 4,352,883 describes the ionic
cross-linking of alginate with divalent cations, in water,
at room temperature, to form a hydrogel matrix.

Tissue precursor cells are mixed with an alginate
15 solution, the solution is delivered to an already implanted
support structure and then solidifies in a short time due to
the presence in vivo of physiological concentrations of
calcium ions. Alternatively, the solution is delivered to
the support structure prior to implantation and solidified
20 in an external solution containing calcium ions.

In general, these polymers are at least partially
soluble in aqueous solutions, e.g., water, or aqueous
alcohol solutions that have charged side groups, or a
monovalent ionic salt thereof. There are many examples of
25 polymers with acidic side groups that can be reacted with
cations, e.g., poly(phosphazenes), poly(acrylic acids), and
poly(methacrylic acids). Examples of acidic groups include
carboxylic acid groups, sulfonic acid groups, and
halogenated (preferably fluorinated) alcohol groups.
30 Examples of polymers with basic side groups that can react
with anions are poly(vinyl amines), poly(vinyl pyridine),
and poly(vinyl imidazole).

Polyphosphazenes are polymers with backbones consisting of nitrogen and phosphorous atoms separated by alternating single and double bonds. Each phosphorous atom is covalently bonded to two side chains. Polyphosphazenes that can be used have a majority of side chains that are acidic and capable of forming salt bridges with di- or trivalent cations. Examples of acidic side chains are carboxylic acid groups and sulfonic acid groups.

Bioerodible polyphosphazenes have at least two differing types of side chains, acidic side groups capable of forming salt bridges with multivalent cations, and side groups that hydrolyze under *in vivo* conditions, e.g., imidazole groups, amino acid esters, glycerol, and glucosyl. Bioerodible or biodegradable polymers, i.e., polymers that dissolve or degrade within a period that is acceptable in the desired application (usually *in vivo* therapy), will degrade in less than about five years and most preferably in less than about one year, once exposed to a physiological solution of pH 6-8 having a temperature of between about 25°C and 38°C. Hydrolysis of the side chain results in erosion of the polymer. Examples of hydrolyzing side chains are unsubstituted and substituted imidizoles and amino acid esters in which the side chain is bonded to the phosphorous atom through an amino linkage.

Methods for synthesis and the analysis of various types of polyphosphazenes are described in U.S. Patent Nos. 4,440,921, 4,495,174, and 4,880,622. Methods for the synthesis of the other polymers described above are known to those skilled in the art. See, for example Concise Encyclopedia of Polymer Science and Engineering, J.I. Kroschwitz, editor (John Wiley and Sons, New York, NY, 1990). Many polymers, such as poly(acrylic acid), alginates, and PLURONICS_{TM}, are commercially available.

Water soluble polymers with charged side groups are cross-linked by reacting the polymer with an aqueous solution containing multivalent ions of the opposite charge, either multivalent cations if the polymer has acidic side groups, or multivalent anions if the polymer has basic side groups. Cations for cross-linking the polymers with acidic side groups to form a hydrogel include divalent and trivalent cations such as copper, calcium, aluminum, magnesium, and strontium. Aqueous solutions of the salts of these cations are added to the polymers to form soft, highly swollen hydrogels.

Anions for cross-linking the polymers to form a hydrogel include divalent and trivalent anions such as low molecular weight dicarboxylate ions, terephthalate ions, sulfate ions, and carbonate ions. Aqueous solutions of the salts of these anions are added to the polymers to form soft, highly swollen hydrogels, as described with respect to cations.

For purposes of preventing the passage of antibodies into the hydrogel, but allowing the entry of nutrients, a useful polymer size in the hydrogel is in the range of between 10,000 D and 18,500 D. Smaller polymers result in gels of higher density with smaller pores.

Temperature-Dependent Hydrogels

Temperature-dependent, or thermosensitive, hydrogels can be used in the methods of the invention. These hydrogels have so-called "reverse gelation" properties, i.e., they are liquids at or below room temperature, and gel when warmed to higher temperatures, e.g., body temperature. Thus, these hydrogels can be easily applied at or below room temperature as a liquid and automatically form a semi-solid gel when warmed to body temperature. As a result, these gels are especially useful when the support structure is first

implanted into a patient, and then filled with the hydrogel-cell composition. Examples of such temperature-dependent hydrogels are PLURONICS™ (BASF-Wyandotte), such as polyoxyethylene-polyoxypropylene F-108, F-68, and F-127, poly (N-isopropylacrylamide), and N-isopropylacrylamide copolymers.

These copolymers can be manipulated by standard techniques to affect their physical properties such as porosity, rate of degradation, transition temperature, and degree of rigidity. For example, the addition of low molecular weight saccharides in the presence and absence of salts affects the lower critical solution temperature (LCST) of typical thermosensitive polymers. In addition, when these gels are prepared at concentrations ranging between 5 and 25% (W/V) by dispersion at 4°C, the viscosity and the gel-sol transition temperature are affected, the gel-sol transition temperature being inversely related to the concentration. These gels have diffusion characteristics capable of allowing cells to survive and be nourished.

U.S. Pat. No. 4,188,373 describes using PLURONIC™ polyols in aqueous compositions to provide thermal gelling aqueous systems. U.S. Pat. Nos. 4,474,751, '752, '753, and 4,478,822 describe drug delivery systems which utilize thermosetting polyoxyalkylene gels; with these systems, both the gel transition temperature and/or the rigidity of the gel can be modified by adjustment of the pH and/or the ionic strength, as well as by the concentration of the polymer.

In one example, a 23% PLURONIC gel can be prepared that is a viscous liquid at below 15°C and above 50°C, and is a hydrogel at temperatures between 15 and 50°C.

pH-Dependent Hydrogels

Other hydrogels suitable for use in the methods of the invention are pH-dependent. These hydrogels are liquids

at, below, or above specific pH values, and gel when exposed to specific pHs, e.g., 7.35 to 7.45, the normal pH range of extracellular fluids within the human body. Thus, these hydrogels can be easily delivered to an implanted support
5 structure as a liquid and automatically form a semi-solid gel when exposed to body pH. Examples of such pH-dependent hydrogels are TETRONICS™ (BASF-Wyandotte) polyoxyethylene-polyoxypropylene polymers of ethylene diamine, poly(diethyl aminoethyl methacrylate-g-ethylene
10 glycol), and poly(2-hydroxymethyl methacrylate). These copolymers can be manipulated by standard techniques to affect their physical properties.

Light Solidified Hydrogels

Other hydrogels that can be used in the methods of
15 the invention are solidified by either visible or ultraviolet light. These hydrogels are made of macromers including a water soluble region, a biodegradable region, and at least two polymerizable regions as described in U.S. Patent No. 5,410,016. For example, the hydrogel can begin
20 with a biodegradable, polymerizable macromer including a core, an extension on each end of the core, and an end cap on each extension. The core is a hydrophilic polymer, the extensions are biodegradable polymers, and the end caps are oligomers capable of cross-linking the macromers upon
25 exposure to visible or ultraviolet light, e.g., long wavelength ultraviolet light.

Examples of such light solidified hydrogels include polyethylene oxide block copolymers, polyethylene glycol polylactic acid copolymers with acrylate end groups, and 10K
30 polyethylene glycol-glycolide copolymer capped by an acrylate at both ends. As with the PLURONIC™ hydrogels, the copolymers comprising these hydrogels can be manipulated by standard techniques to modify their physical properties

such as rate of degradation, differences in crystallinity, and degree of rigidity.

Tissue Precursor Cells

5 Tissue precursor cells can be obtained directly from a mammalian donor, e.g., a patient's own cells, from a culture of cells from a donor, or from established cell culture lines. The mammal can be a mouse, rat, rabbit, guinea pig, hamster, cow, pig, horse, goat, sheep, dog, cat, and the mammal can be a human. Cells of the same species
10 and preferably of the same immunological profile can be obtained by biopsy, either from the patient or a close relative. In the case of neural stem cells isolated from nervous tissue, they can be isolated from healthy tissue adjacent defective tissue, or from other sites of healthy
15 tissue in the mammal.

Using standard cell culture techniques and conditions, the cells are then grown in culture until confluent and used when needed. The cells are preferably cultured only until a sufficient number of cells have been
20 obtained for a particular application.

If cells are used that may elicit an immune reaction, such as human muscle cells from an immunologically distinct donor, then the recipient can be immunosuppressed as needed, for example, using a schedule of steroids and
25 other immunosuppressant drugs such as cyclosporine. However, the use of autologous cells will avoid such an immunologic reaction.

Cells can be obtained directly from a donor, washed, suspended in a selected hydrogel before being delivered into
30 a support structure. To enhance cell growth, the cells are added or mixed with the hydrogel just prior to insertion into the support structure.

Cells obtained by biopsy are harvested, cultured, and then passaged as necessary to remove contaminating, unwanted cells. The isolation of chondrocytes and osteoblasts is described in the examples below. The isolation, culturing, and passaging of central nervous system stem cells, e.g., brain, spinal cord, autonomic nervous system cells, and neuroendocrine cells (that are maintained in an undifferentiated state) is also described below.

Cell viability can be assessed using standard techniques including visual observation with a light or scanning electron microscope, histology, or quantitative assessment with radioisotopes. The biological function of the cells delivered to the support structure can be determined using a combination of the above techniques and standard functional assays.

Examples of cells that can be delivered to the support structure and subsequently grow new tissue include epidermal cells; chondrocytes and other cells that form cartilage ("cartilage-forming cells"); macrophages; dermal cells; muscle cells; hair follicles; fibroblasts; organ cells; macrophages; osteoblasts, periosteal cells, and other cells that form bone ("bone forming cells"); endothelial cells; mucosal cells, e.g., nasal, gastric, bladder and oral mucosal cells; pleural cells; ear canal cells; tympanic membrane cells; peritoneal cells; Schwann cells; corneal epithelial cells; gingiva cells; tracheal epithelial cells; and neural cells, including neural stem cells and neurons, e.g., from the brain, spinal cord, and autonomic nervous system.

Neural stem cells for use in the new compositions and methods can be isolated from a variety of nervous system tissues. For example cells can be isolated from spinal cord

tissue, brain tissue, and other central nervous system tissues, as well as from peripheral nervous tissues, autonomic nervous system tissues. The peripheral nervous tissue stem cells arise from the CNS in the spinal cord gray matter, and can be isolated as described herein for the isolation of neural stem cells from the spinal cord. Post-ganglionic autonomic nervous system stem cells (outside the spinal cord) can be isolated as described herein from various tissues, for example, from any innervated organs, such as heart, kidney, small intestine, liver, lung, and bladder tissues. Pre-ganglionic autonomic nervous system stem cells are located in the gray matter of the spinal cord and therefore are isolated in the same way as, and in conjunction with, spinal cord stem cells.

Neuroendocrine stem cells are found in areas such as the adrenal medulla and differentiate into cells that make catecholamines such as epinephrine and norepinephrine, and also from the pancreas, in which case they develop into cells that secrete insulin (beta cells) or glucagon (alpha cells). Other sources of neuroendocrine cells are the parathyroid, pituitary, and the hypothalamus glands. These cells can be isolated from adult mammals, e.g., humans, in the same manner as post-ganglionic autonomic nervous system stem cells, and can be used to prepare tissue constructs used to treat endocrine diseases such as diabetes.

The isolated cells must be undifferentiated, in that when exposed to various growth factors the cells can differentiated into neurons, oligodendrites, astrocytes, and other nerve tissues. The cells can be isolated from adult nervous tissue, although fetal nervous tissue can also be used.

If the nervous tissue to be repaired or augmented is spinal cord tissue, the nerve cells used in the hydrogel-

cell composition can be isolated from spinal cord tissue. Likewise, hydrogel-neural stem cell compositions to repair brain tissue defects can include neural cells isolated from brain tissue. However, if stem cells obtained from neural
5 tissues are sufficiently undifferentiated, these cells can be used to treat any nervous system defect, because they can be induced to differentiate into the particular kinds nervous tissue required.

The isolated stem cells are maintained in culture,
10 including cytokines such as epidermal growth factor and basic fibroblastic growth factor, in an undifferentiated state by repeated trituration at each passage (e.g., weekly). Once the isolated neural stem cells begin to multiply in culture, they also begin to coalesce to form
15 neurospheres that contain numerous undifferentiated cells. The key to maintaining these cells in an undifferentiated state is to triturate the cells before they begin sending out axons and dendrites. The trituration process should be carried out every 5 to 10 days; weekly intervals appear to
20 be satisfactory. This repeated trituration and passaging enables the isolated, undifferentiated neural stem cells to be cultured indefinitely.

Preparation of Hydrogel-Cell Compositions

First, a hydrogel of choice is prepared using
25 standard techniques. For example, a biodegradable, thermosensitive polymer at a concentration ranging between 5 and 25% (W/V) is useful for the present invention. If the hydrogel is an alginate, it can be dissolved in an aqueous solution, for example, a 0.1 M potassium phosphate solution,
30 at physiological pH, to a concentration between 0.5 to 2% by weight, e.g., 1%, to form an ionic hydrogel.

Second, isolated tissue precursor cells are suspended in the polymer solution at a concentration mimicking that of the tissue to be generated, e.g., between 10 and 100 million cells/ml, for example, between 20 and 50 million cells/ml. The optimal concentration of cells to be delivered into the support structure is determined on a case by case basis, and may vary depending on cellular type and the region of the patient's body into which the support structure is implanted. Optimization experiments require modifying only a few parameters, i.e., the cell concentration or the hydrogel concentration, to provide optimal viscosity and cell number to support the growth of new tissue.

For example, as demonstrated below, new growth of bone tissue in the shape of a rat femur, penetrating into the entire thickness of a polymeric support structure 5 mm in diameter, was observed after injecting a hydrogel-cell composition containing approximately 50 million periosteal cells per ml.

Delivery to and Implantation of the Support Structure

The liquid hydrogel-cell composition is delivered into any of the permeable support structures described herein. Fig. 1 shows a cross-sectional schematic of a filled support structure for the case of a support structure formed from a mesh of interwoven polymer fibers. The spaces between the fibers form interconnected pores, which are filled with the liquid hydrogel-cell composition. Within a short time after delivery, e.g., less than three or five minutes, the hydrogel solidifies, thereby keeping the cells suspended in the hydrogel within pores of support structure. The solidified hydrogel keeps the cells viable, allowing diffusion of nutrients and waste

products through the interconnected pores of the support structure and ultimately resulting in the growth of new tissue and its engraftment to the patient's body.

5 The support structure 10 provides a shape and structure for the solidifying hydrogel-cell composition while still providing permeable paths for diffusion through the hydrogel. The tissue grown by the cells will conform to the shape of the support structure and the support structure will provide structural integrity sufficient to resist
10 external stresses from the environment and surrounding tissues during growth of the new tissue. In certain embodiments, such as when the hydrogels contain nervous system stem cells, the hydrogel plays a key role in keeping the cells in an undifferentiated condition prior to
15 implantation in the body by keeping the cells suspended and not adhered to the support structure, such as the polymer fibers in a mesh structure. After implantation, the support structure plays a role in enhancing the differentiation of the stem cells to become distinct nerve cells such as motor,
20 sensory, and striatal neurons.

The liquid hydrogel-cell composition can be delivered to the shaped support structure either before or after the support structure is implanted into a patient. The specific method of delivery will depend on whether the
25 support structure is sufficiently "sponge-like" for the given viscosity of the hydrogel-cell composition, i.e., whether the support structure easily retains the liquid hydrogel-cell composition before it solidifies. Sponge-like support structures can be immersed within, and saturated
30 with, the liquid hydrogel-cell composition, and subsequently removed from the composition. The hydrogel is then allowed to solidify within the support structure. The hydrogel-cell-containing support structure is then implanted into the

patient. The support structure can also be implanted into the patient before the hydrogel completely solidifies. Alternatively, a sponge-like support structure can be injected with the liquid hydrogel-cell composition, either before or after the support structure is implanted in the patient. The hydrogel-cell composition is then allowed to solidify. The volume of the liquid hydrogel-cell composition injected into the support structure is typically less than, but somewhat comparable to the volume of the support structure, i.e., the volume of the desired tissue to be grown.

Support structures that do not easily retain the liquid composition require somewhat different methods. In these cases, for example, the support structure is immersed within and saturated with the liquid hydrogel-cell composition, which is then allowed to partially solidify. Once the cell-containing hydrogel has solidified to the point where the support structure can retain the hydrogel, the support structure is removed from the partially solidified hydrogel, and, if necessary, partially solidified hydrogel that remains attached to the outside of the support structure is removed, e.g., scraped off the structure. Alternatively, the liquid hydrogel-cell composition can be delivered into a mold containing the support structure. For example, the liquid hydrogel-cell composition can be injected into an otherwise fluid-tight mold that contains the support structure and matches its outer shape and size. The hydrogel is then solidified within the mold, e.g., by heating, cooling, light-exposure, or pH adjustment, after which time the hydrogel-cell-containing support structure is removed from the mold and is ready to be implanted into a patient.

In other embodiments, the support structure is implanted into the patient, e.g., subcutaneously or subdermally, and thereafter, the liquid hydrogel-cell composition is delivered to implanted support structure, for example by injection through a syringe or endoscopic instrument. The volume of hydrogel-cell composition injected into the support structure should approximate the size of the support structure (i.e., the volume displaced by the desired tissue to be grown). The implanted support structure provides space and a structural template for the injected liquid hydrogel-cell composition. As described above, some of the hydrogel-cell composition may leak from the support structure prior to solidifying. However in this embodiment, existing tissue (e.g., muscle, bone, cartilage) surrounding the implanted support structure would sufficiently constrain the liquid hydrogel-cell composition within the support structure until it gels. In other embodiments, such as a tissue replacement for skin, the support structure can be externally attached to the patient, e.g., by suturing or adhesives.

In any of the above cases, the hydrogel is solidified using a method that corresponds to the particular hydrogel used (e.g., gently heating a composition including a PLURONIC™ temperature-sensitive hydrogel).

To implant the support structure, the implantation site of the mammalian patient can be exposed by surgical resection and the support structure implanted directly at that site. Alternatively, the implantation site can be viewed with the aid of, e.g., an endoscope, laparoscope, arthroscope, or esophagoscope, all of which can be modified to include a mechanical articulation and delivery system for implanting the support structure through a small incision. During implantation, the site is cleared of bodily fluids

including blood, e.g., with a burst of air or suction. Thus, the hydrogel-cell-containing support structure can be introduced through a laparoscope, endoscope, laryngoscope, cystoscope, proctoscope, or thoracoscope to any the interior
5 surface of any lumen or cavity, or other surfaces, such as intraperitoneal, extraperitoneal, and thoracic cavity, and then implanted into the desired space.

Throughout the implantation procedure, the amount of trauma caused to the cells during the delivery and
10 implantation steps can be determined by measuring a biological function specific for the cells being used. For example, when chondrocytes are being applied, the integrity of the new cartilage can be evaluated by standard biomechanical stress analyses, such as determination of
15 compression moduli.

It is important to note that in addition to providing a direction and pathway for the growth of new tissue, the support structure can also alter external stresses imparted to the developing tissue. In particular,
20 the support structure can transmit or relieve external stresses along selected directions, which can optimize tissue development. For example, the support structure can resist compressive forces, and thereby relieve compressive forces on bone tissue developing within the support
25 structure. At the same time, the new bone cells develop under normal stresses, e.g., those produced by walking, thereby forming stronger bone tissue than bone tissue grown absent any load forces.

Also, the support structure can transmit external
30 tensile forces imparted by stretching or twisting motions to developing tendon or ligament tissue. Such tensile forces promote the growth of tendon and ligament tissue and

generate tissue that is stronger than tissue grown absent such forces.

The positioning of the support structure within the patient and the internal construction of the support structure determines how the support structure will couple external forces to the developing tissue. For example, polyglycolic fibers used to construct a support structure can be oriented along a preferred direction so that the mechanical properties of the support structure are anisotropic. In such examples, the anisotropic properties of the support structure may affect the development of different fascial planes, the orientation of bundles in tissue, such as muscular bundles in skeletal tissue and cardiac muscle, the conducting system within the cardiac muscle, and the development of polar tissues such as neurons.

Applications

Since the hydrogel-cell compositions can support many different kinds of precursor cells and the support structures can guide the development and shape of the new tissue, the new methods can be used in any instance in which it desirable to generate new tissue. Particular applications that are described below relate to the generation of cartilage, bone, and neural tissues.

Treatment of Cartilage Defects

Cartilage is a specialized type of dense connective tissue consisting of cells embedded in a matrix. There are several kinds of cartilage. Hyaline cartilage is a bluish-white, glassy translucent cartilage having a homogeneous matrix containing collagenous fibers which is found in articular cartilage, in costal cartilages, in the septum of the nose, and in the larynx and trachea. Articular

cartilage is hyaline cartilage covering the articular surfaces of bones. Costal cartilage connects the true ribs and the sternum. Fibrous cartilage contains collagen fibers. Yellow cartilage is a network of elastic fibers holding cartilage cells which is found primarily in the epiglottis, the external ear, and the auditory tube. By harvesting the appropriate chondrocyte precursor cells, any of these types of cartilage tissue can be grown using the methods of the invention.

For example, new tissue can be grown for a cartilage meniscus replacement in the knee. A biocompatible support structure having physical characteristics similar to, for example, a sponge, is cut or molded into the shape of the meniscus to be replaced. The support structure, which can also be biodegradable, is then delivered via an arthroscope into the knee joint and positioned in the proper orientation. Since the support structure is sponge-like, it can be compressed as it is being delivered through the arthroscope and will re-expand when properly positioned in the knee. Thereafter, a liquid hydrogel-chondrocyte composition is injected into the sponge, filling the pores of the support structure. The hydrogel subsequently solidifies, taking the shape of the desired meniscus replacement and providing a suspension for the chondrocytes that permits diffusion of nutrients and waste products to and from the suspended chondrocytes. Over time, e.g., over a period of approximately six weeks, the suspension will become vascularized and the chondrocytes will grow new cartilaginous tissue that takes the shape of the meniscus and engrafts to existing tissue.

Treatment of Bone Defects

In another example, periosteal cells (i.e., bone-growing cells) can be used in the invention to fill bone

defects or to prepare entire new bones. First, the support structure is sized to the dimensions of the bone defect. The support structure can be formed from polymer fibers and cut or molded into the desired size and shape, or
5 alternatively, the support structure can be formed from a piece of hydroxyapatite or coral and machined into the desired size and shape. The hydrogel-periosteal-cell composition can then be delivered to the support structure either before or after it is positioned within the bone
10 defect. If necessary, adhesives can be used to adhere the support structure to the bone within the bone defect. Once again the hydrogel solidifies and suspends and maintains the cells, which subsequently grow bone tissue and fill in the bone defect. In some cases, the support structure allows
15 some partial weight bearing even before the new bone tissue is completely grown.

Treatment of Nervous Tissue Defects

In other examples, the hydrogel-cell composition can include cells that generate central nervous tissue,
20 including brain tissue, spinal cord tissue, peripheral nerves, autonomic nerves, and neuroendocrine cells. The support structure can be implanted into a patient having a spinal cord or brain defect following a stroke or other injury, and the hydrogel-cell composition can be injected
25 into the support structure, or the support structure can be permeated with the hydrogel-cell composition and then implanted. The support structure then guides the growth and development of the newly-generated neural tissue. The defect in the nervous tissue may result, for example, from a
30 disease, physical trauma, reperfusion injury, ischemia, or infection. However, any defect in nervous tissue can be treated using the new methods.

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The new hydrogel-neural stem cell compositions can be used to treat defects in nervous tissue throughout the body. For example, infarcted brain tissue, damaged or severed nerve tissues in the spinal cord, or autonomic
5 nerves, can all be treated by replacing the damaged tissue with the new hydrogel-neural stem cell composition, with or without a support structure, as required.

Initially, the area and boundaries of the defective nervous tissue is defined, for example, using MRI, CAT scan,
10 PET scan, evoked electrical potentials, or blood flow analysis. Once the outer boundaries of the defective, i.e., damaged or injured nervous tissue are defined, the damaged or injured tissue is surgically removed with a clean cut to create a cavity, leaving normal healthy nervous tissue at
15 the boundaries of the cut (the edges or surfaces of the cavity). Grey matter cells are removed from the healthy tissue surrounding the damaged tissue and cultured to produce isolated neural stem cells that are cultured, expanded, and passaged to provide a sufficient number of
20 cells for treatment.

While the isolated neural stem cells are being cultured and expanded, e.g., for a 7 to 14 day period, an inert, biocompatible spacer (e.g., of calcium alginate) that is designed to induce no or minimal inflammatory responses,
25 is preferably inserted into the space created by removal of the damaged nervous tissue. This biocompatible spacer can be permeated with drugs such as growth factors, anti-inflammatory compounds such as steroids, and/or other pharmaceutical agents designed to prevent fibrous tissue
30 from growing into the space created by the removal of the damaged tissue.

Once the autologous cells have been expanded to a sufficient number of cells, the hydrogel-cell composition is

prepared as described herein and is added to a support structure, e.g., a fibrous mesh, formed into the shape of the removed damaged tissue. The support structure loaded with the hydrogel-cell composition is then implanted into the space remaining after the spacer is removed. Of course, if cultured neural stem cells are available for immediate implantation, no spacer is required. Alternatively, the support structure can be implanted and then loaded with the hydrogel-neural stem cell composition, e.g., with a syringe.

Although it is preferred that surgery occur as soon after the injury to the nervous system tissue as possible, the new methods can be used to treat injuries that occur several weeks to months prior to surgery, as long as the surgery leaves a clean cut exposing healthy nervous tissue on either side of the implanted support structure.

Autologous cells can also be removed from sites other than the surgery site where the damaged nervous tissue is removed. For example, autologous nervous cells can be removed using stereotactic biopsies, or from aspirates using needles, e.g., inserted into the spinal cord. This is possible because only a very small number of isolated neural stem cells, e.g., about five cells, are required for the new culturing methods. For example, a 22 gauge aspiration needle can be used.

Any localized damage or trauma to nervous system tissue can be repaired, including infarcts, tissue damage caused by cancer, stroke, or wounds such as gunshot wounds or other crushing or slicing injuries. Furthermore, various nervous system diseases such as Parkinson's disease can be treated by implanting hydrogel-cell compositions including neural stem cells engineered to include a gene that expresses a protein that is missing in the diseased patient.

Treatment of Autonomic Nervous System Defects

The autonomic nervous system (ANS), which includes the sympathetic and parasympathetic nervous systems, is divided into pre- and post-ganglionic neurons. The pre-ganglionic neuron cell bodies are located in the central nervous system and neural stem cells that form these pre-ganglionic neurons are isolated using the techniques for CNS stem cell isolation described herein. A technique for isolating post-ganglionic autonomic nervous system neural stem cells is described in Example 8.

The new hydrogel-ANS neural stem cell compositions can be used to innervate virtually any organ system of the body by first isolating these cells from the host, and expanding them in culture using cytokines and hormones such as EGF and bFGF with standard complete culture media (Dulbeccos Modified Eagle Medium (DMEM)/F12) in an incubator. With appropriate expansion of the cell line suspension in a biologically inert hydrogel such as PLURONIC F127, a surfactant, a concentration of from 5 to 50 x 10⁶/ml can be achieved. This hydrogel composition is then injected directly (locally) into a dysfunctional organ of the host (e.g., an atonic bladder wall or an aganglionic segment of bowel). Over time, the hydrogel would disappear, leaving behind ANS neural stem cells that differentiate into autonomic neurons that would naturally synapse (connect) with existing neurons, resulting in restoration of function of the organ.

Clinical applications would include any organ system or portion of the body that is innervated by the autonomic nervous system. Examples include: atonic bladders in diabetics and the elderly, aganglionic bowel disturbances such as Hirschsprung's Disease, sexual dysfunction where impotence is caused by innervation disturbances, autonomic

blood flow problems to the skin or any organ, and actual innervation defects of blood vessels. Additionally, glandular dysfunction because of loss of autonomic innervation can be treated.

5 In addition, ANS stem cells can be used in conjunction with other engineered tissues, for example, in engineered organs, to guide cell organization and autonomic innervation of the newly generated tissues, e.g., in the liver, kidney, bowel, and bladder. These engineered organs
10 would include a support structure and a hydrogel-cell composition in which the tissue precursor cells include both the organ cells, e.g., hepatocytes or cardiac muscle precursor cells, and autonomic, post-ganglionic neural stem cells.

15 Additional Uses of Neural Stem Cells

Neural stem cells isolated from the CNS, e.g., the cortex, striatum, and spinal cord, as well as the ANS, can also be used as "smart cells" that assist in orchestrating the organization and development of other engineered
20 tissues, for example, in artificial organs such as the kidneys, liver, bowel, and bladder, by including these neural stem cells in a mix of precursor cells used to prepare tissue engineered organs.

Neurodegenerative processes such as Parkinson's
25 disease can be treated with autologous neural stem cells that are genetically engineered using standard techniques, for example as described in U.S. Patents Nos. 5,082,670 and 5,762,926, to produce dopamine (or with naturally occurring stem cells that make dopamine), and then added to a hydrogel
30 and/or injected into a support structure. Such hydrogel-neural stem cell compositions will function longer after implantation than an injected suspension of cells.

Similarly, genetic neurodegenerative processes, such as the leukodystrophies, can be treated with neural stem cells that have been genetically repaired.

5 In addition, neuroendocrine stem cells can be isolated from the adrenal medulla or the pancreas and used to prepare endocrine tissue constructs to treat such endocrine disorders such as diabetes.

EXAMPLES

10 The present invention will be further understood by reference to the following non-limiting examples.

Example 1

Preparation of a Calcium-Alginate-Chondrocyte Composition

A calcium alginate mixture is obtained by combining
15 calcium sulfate, a poorly soluble calcium salt, with a 1% sodium alginate dissolved in a 0.1 M potassium phosphate buffer solution (pH 7.4). The mixture remains in a liquid state at 4°C, and until its temperature is increased to over 15°C. Chondrocytes are isolated from cartilage tissue,
20 e.g., the articular surface of calf forelimbs, using standard techniques, and are added to the mixture to generate a final cellular density of about 2 to 5 x 10⁷/ml (e.g., representing approximately 10% of the cellular density of human juvenile articular cartilage). Such a
25 mixture can be delivered into a permeable support structure.

Example 2

Preparation of a Thermosensitive Hydrogel-Chondrocyte Composition

A biocompatible, biodegradable, reverse-
30 thermosensitive copolymer gel was obtained by preparing a

30% weight/volume solution of a PLURONIC™ F127, F68 block copolymer (available from BASF). The solution remains in a liquid state at less than 15°C, and solidifies within 5 to 10 minutes as the temperature is increased to over 15°C.

- 5 Chondrocytes were isolated from the articular surface of calf forelimbs using standard techniques, and added to the hydrogel mixture to generate a final cellular density of about $2 \text{ to } 6 \times 10^7/\text{ml}$. Such a mixture can be delivered into a permeable support structure.

10 Example 3

Guided Tissue Growth of a Distal Femur in a Rat Using a Permeable Support Structure

- In this example, a synthetic, biocompatible polymer support structure was used to guide the development of new
15 bone tissue formed from a hydrogel-cell composition to replace the distal femur in a rat.

- A rat was sacrificed and its distal femur removed. The bone was immersed into liquid in a graduated cylinder to measure the volume displaced by the bone, which was
20 approximately 1 cc. Thereafter, an external mold of the femur was made using standard acrylic molding techniques. Using the external mold, a porous support structure composed of polyglycolic and polylactic acid was created as follows.

- Polyglycolic acid fibers, 14 microns in diameter,
25 were entangled into a mesh in which the fibers are space from one another by an average of 150-250 microns. The fiber mesh was then immersed in a 1% solution of polylactic acid for about 10 seconds, when it became saturated. The solution, which has a viscosity similar to water, coated the
30 fibers and welded the fibers to one another where the fibers intersected one another. While the fiber mesh was still wet with the polylactic acid solution, it was compressed in the

mold and allowed to dry for several minutes. Once the solution evaporated and the polylactic acid dried, the fiber mesh retained the shape of the mold. The procedure took place at room temperature and produced a support structure in the shape of the distal femur with holes or pores approximately 200 microns in diameter. The femur itself was approximately 15 mm long and between 3-7 mm in diameter. Fig. 2 is a photograph of the femur support structure.

The support structure was then implanted subcutaneously onto the back of a nude mouse. Fig. 3 shows a photograph of the nude mouse after the support structure was implanted. The photograph clearly shows the definition of the femur-shaped support structure through the skin of the mouse. A hydrogel-cell composition containing periosteal cells was then injected into the structural support.

The periosteal cells were isolated by obtaining strips (approximately 5 mm by 3 mm) of periosteum surgically removed from the surface of a bone. The strips of periosteum were placed into a tissue culture dish containing nutrient, and periosteal cells were shed from the periosteum strips and multiplied over a period of time. The cells were held in an incubator *in vitro* and allowed to multiply for up to 4 weeks. The hydrogel used in this example was calcium alginate, whose preparation is described in Example 1. The periosteal cells were added to the calcium alginate to produce a hydrogel-cell composition having a concentration of approximately 50 million cells per cc. A low concentration of calcium was also added to the composition.

A volume of the hydrogel-cell composition equal to the remeasured volume of the distal femur bone (approximately 1 cc) was injected through a 25-gauge needle using a 3 cc syringe into the support structure implanted

under the skin of the nude mouse. The hydrogel solidified following ionic cross-linking with calcium ions, which are present in all body tissue but were also added to the hydrogel-cell composition prior to injection. After
5 solidification of the hydrogel, the support structure allowed the cell-containing hydrogel suspension to maintain its shape and resist compressive forces while new tissue developed.

After 8 weeks, the mouse was sacrificed and the
10 femur-shaped support structure was removed for examination. As shown in the photograph in Fig. 4, new tissue engrafted to the support structure. Histological examination of the support structure, as shown in the photograph in Fig. 5, indicated new tissue growth and vascularization as indicated
15 by the appearance of a Haversian system in the new bone. In particular, Fig. 5 shows a portion of the new tissue in which bone cells (shown in light) surround a blood vessel (shown in dark) at the center of the photograph. Additional bone cells filled the entire support structure. The
20 photograph was taken through a 20 X microscope objective and the photograph correspond to a 640 micron by 950 micron cross-sectional area.

Example 3 demonstrates that implantation of a permeable support structure having a defined shape guides
25 the shape and development of new tissue formed from a hydrogel-cell composition injected into the support structure. The support structure resists mechanical and compressive forces and allows the cell hydrogel composition to develop new tissue of the desired shape and volume. The
30 mouse model in this example provides evidence that the same techniques will be effective to generate new tissue in human patients.

Example 4

Isolation of Spinal Cord Stem Cells

Spinal cord stem cells were isolated from Fisher rats. The rats were decapitated and the bodies put on ice for fifteen minutes. The spinal cord was removed and placed into cold phosphate buffered saline (PBS) at 40°F in petri dishes. Spinal cords were gently opened to expose gray matter with a number 15 scalpel, and the gray matter was scraped from the remaining white matter tissue. The white matter was removed with forceps, leaving grey matter cells suspended in the PBS. The PBS was stirred and poured into a centrifuge tube half filled with cold PBS. The liquid was centrifuged at 1200 rpm x 5 minutes, and the supernatant was suctioned off.

Ten centiliters of 0.05 percent trypsin were added to the tube of cells and incubated for 5 minutes at 37°C in a water bath. This process of enzyme degradation breaks down tissue debris and kills the majority of the cells (differentiated cells) in the solution except for the spinal cord stem cells.

After five minutes, the trypsin was inactivated with DMEM (10% fetal calf serum albumin). The mixture was stirred and triturated using a regular bore Pasteur pipette to break up the cells into as fine a mixture as possible. Again, the goal was to destroy all of the cells other than the stem cells. The cells were further triturated with a fine microcapillary having an inner diameter of approximately 100 microns. This process kills mature nerve cells by breaking appendages and processes. After the second trituration, the cells were spun again at 1200 rpm x 5 minutes. The supernatant was decanted and the remaining cells were suspended in complete Dulbeccos Modified Eagle Medium (DMEM)/F12 (Gibco, Gaithersburg, MD). Complete

medium includes glucose, transferrin, insulin, putrescine, selenium, penicillin-streptomycin, and HEPES buffer in standard concentrations. Progesterone was added at a concentration of 20 ng/ml.

5 To this culturing medium were added epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) at a concentration of 20 nanograms per ml. The same growth medium can be used for isolating and expanding human spinal
10 cord stem cells. To isolated stem cells from brain tissue of animals or humans, the same culture medium can be used (DMEM), but is supplemented only with EGF, not bFGF.

 The isolated stem cells were cultured in 20 ml of the supplemented growth medium at 37°C in a flask with 5 percent CO₂. Five mls of fresh complete DMEM/F12
15 supplemented as described above were added every three days. As the cells multiply (expand) they are passaged by pipetting the cells out of the flask, spinning them at 1200 rpm x five minutes and resuspending in fresh supplemented media (20 ml) and triturating with a regular bore pipette
20 (and optionally with a microcapillary pipette).

 In each passage, the cells in one flask are divided into two equal portions, and resuspended in two separate flasks of 20 mls each. The first passage (after about 7 days of culturing) yields approximately 1 to 6 million
25 cells, the second passage a week later yields approximately 2 to 12 million cells. At three to four weeks, the population of cells stops growing, and is maintained at a constant level. As long as the cells are passaged and triturated on a weekly basis, the cells can be cultured
30 indefinitely in an undifferentiated state.

Example 5

Preparation of a Hydrogel-Spinal Cord Stem Cell Composition

Approximately 20 to 50 million cells per ml of the spinal cord stem cells isolated as described in Example 4 were suspended in a solution of complete DMEM/F12 supplemented with EGF, bFGF, and a 23% concentration of PLURONIC™ F127. This particular PLURONIC is a surfactant that causes the culture medium to gel at between 15 and 50°C, and to become a viscous liquid below 15°C and above 50°C.

The hydrogel-spinal cord stem cell composition was then used to permeate a non-woven mesh of polyglycolic acid (PGA) fibers that have an individual diameter of about 15 microns. This mesh has the appearance of a small mass of cotton, and was permeated by dipping the mesh into the liquid hydrogel-cell composition. Alternatively, the liquid hydrogel can be injected into the nonwoven mesh.

The PGA mesh holds the PLURONIC gel by capillary action and provides guidance for the growth of the neural stem cells once implanted into an animal. Furthermore, the PGA fibers stimulate the differentiation of the undifferentiated neural stem cells.

Once permeated, a small plug of PGA nonwoven mesh having a diameter of approximately 3 millimeters and a length of approximately 4 to 5 millimeters, contained approximately 50 microliters of the hydrogel-cell composition, and thus contained approximately 1 to 5 million cells.

Example 6

Repair of Spinal Cord Injury in Rats

Thirty Fisher rats were anesthetized and their spinal cords were surgically injured. In particular, in

each case, the spinal cord was totally severed in two places approximately 3 to 4 millimeters apart, and the intermediate section of the spinal cord was removed, leaving a gap of 3 to 4 millimeters. This gap was confirmed by magnetic
5 resonance imaging one week after surgery. The hydrogel-stem cell composition prepared as described in Example 5 was used to replace the portion of the spinal cord that had been removed in fifteen experimental animals. Fifteen controls
10 had the same surgery in which a portion of the spinal cord was removed, leaving a 3 to 4 mm gap, but no hydrogel-cell composition was inserted to replace the gap. The wounds over the spinal cord were sutured in all animals. The vertebrae of the spinal column remained stable, because only the lamina and posterior processes were removed.

15 Of the original 30 rats, 21 died from surgery or complications, such as spinal shock, infection, or dehydration, leaving nine surviving rats. Of these nine rats, five were experimental, and four were controls.

All of the living rats received standard post-
20 operative care, and were observed over time and fed a normal diet. The rats' overall sensation and hind limb function was monitored in two ways. First, pain sensation was monitored by pin prick (right and left hind leg, right and left thigh, back, and abdomen) to monitor head movement,
25 which indicates awareness of the pain, rather than a mere reflex, which is indicated, for example, by limb withdrawal. Second, motor function was evaluated for both reflex and spontaneous purposeful motor function. After six weeks, the five control rats showed no pain sensation and no purposeful
30 movements of the hind limbs. The hind limbs were initially flaccid, but then developed spastic paralysis after approximately 7 to 10 days.

In contrast, the four experimental rats showed a clear return of function of varying degrees in their hind limbs. The rats showed an awareness of pain in all sites (left and right hind legs and thighs, back, and abdomen), and showed clear spontaneous purposeful motor function. Based on visual observations, the best of the four experimental rats showed approximately 90 percent function in the right hind leg and approximately 80 percent function in the left hind limb after four weeks. This rat in fact raised its entire body on its two hind legs to reach for food and water. After four weeks, the two moderately recuperating rats showed approximately 50 to 60 percent function in one hind limb and approximately 25 percent function in the other hind limb. The "worst" of the four experimental rats showed approximately 30 percent function in the right hind leg and approximately 10 to 15 percent function in the left hind leg. All of the experimental rats continued to improve.

Example 7

20 **Culturing of Isolated Neural Stem Cells in Hydrogel**

In this study neural stem cells were suspended in PLURONIC® F127, to demonstrate the *in vitro* survival, optimal cell density for culture, and phenotypic expression in response to various factors including epidermal growth factor (EGF), nerve growth factor (NGF), and 10% fetal bovine serum (FBS).

Striatal stem cells were harvested from the striata of day 15 Balb-C mouse embryos (as described in Example 5), and incubated at 37°C in complete DMEM/F12 (1:1) with progesterone (as a trophic agent) and EGF. Cells were suspended in the above media containing 23% PLURONIC F127, at 5, 10, and 20 million cells/ml, and incubated at 37°C in

5% CO₂ for 21 days. Cells harvested in the same manner from the same sources were suspended at a concentration of 5 million cells/ml in the absence (control) or presence of EGF 20ng/ml, NGF 100ng/ml, or 10% FBS, and incubated in the same fashion. At days 7 and 14, cells were removed from culture, deposited onto slides via cytopspin, and probed with antibodies to GFAP (glial fibrillar acidic protein), NF (neural filament), and O4 as markers for glia, neurons, and oligodendrocytes, respectively.

No difference in cell growth was observed between different cell concentrations. In addition, pluripotentiality was exhibited by differentiation into a wide range of CNS cells, as indicated by positive antibody staining for GFAP, NF, and O4. As shown in the Table below, phenotypic regulation is demonstrated by the increase in NF+ cells in the NGF and FBS groups at day 14. This is consistent with regulation of lineage formation of neural stem cells observed in suspension culture.

Table

Neurosphere/Pluronic Ab staining			
DAY 7	GFAP+	NF+ *	O4+
20 ng/mL EGF	40/100	2/100	12/100
100 ng/mL NGF	25/100	1/100	1/100
no factors	25/100	1/100	0/100
DAY 14	GFAP+	NF+	O4+
20 ng/mL EGF	33/100	2/100	10/100

100 ng/mL NGF	16/100	7/100	7/100
no factors	24/100	2/100	10/100
10% FBS	18/100	6/100	15/100

Based on these experiments, it is clear that neural stem cells survive and proliferate when cultured in PLURONIC F127 at a concentration of 5 million cells/ml. The phenotypic expression of these stem cells was controlled by growth factors in a similar fashion as stem cells cultured in media alone.

In another experiment, adult rat spinal cord stem cells were suspended in PLURONIC® 127 with a PGA mesh as described in Example 5. Cells not used for implantation in Example 6 were incubated at 37°C with normal air and 5% CO₂. The stem cells formed neurospheres and attached to the PGA mesh and survived for one week before being discarded.

Example 8

Isolation of Post-Ganglionic Autonomic Nervous System Stem Cells from the Heart, Lung, Liver, Kidney, Bladder and Intestine of the Adult Rat

An adult rat was sacrificed by decapitation and the whole body was cooled on ice. Under sterile conditions, the heart, lung, liver, kidney, bladder, and intestines, were dissected out and a 5 mm fragment of tissue from each organ was placed in a separate petri dish of cold PBS. Each tissue fragment was aggressively scraped with a # 15 scalpel blade into cold PBS. The scraped tissue fragments were discarded and the remaining PBS was added to 10 ml of cold PBS and centrifuged at 1200 rpm for 5 minutes.

The PBS was suctioned off and 10 ml of 0.05% trypsin was added to the sediment and incubated in a 37°C water bath for 5 minutes. The trypsin was inactivated by adding 10 ml of DMEM + 10% FSA. The specimens were triturated with a
5 Pasteur pipette until the tissue was in a fine suspension, then triturated again with a reduced bore Pasteur micropipette until the tissue was dissociated. The specimens were centrifuged at 1200 rpm for 5 minutes. The sediment was resuspended in DMEM/F12 supplemented with 20
10 ng/ml of EGF and bFGF and placed in an incubator at 37°C with 5% CO₂. All specimens, from each organ, contained many small, round neural stem cells. Rare differentiation into neurons was seen with cells that attached to the bottoms of the flasks.

15 This same procedure can be used to extract and isolate ANS neural stem cells from any organ of the body with autonomic innervation.

Example 9

20 Isolation of Neuroendocrine Stem Cells from the Adrenal Gland and the Pancreas of the Adult Rat

An adult rat was sacrificed by decapitation and the whole body was cooled on ice. Under sterile conditions, the adrenal gland and pancreas were dissected out to obtain a 5 mm fragment of tissue. The adrenal gland tissue fragment
25 was aggressively scraped with a # 15 scalpel blade into cold PBS. The pancreas tissue fragment was scraped with a # 15 scalpel into cold DMEM-F12 medium and 10% FSA, which has the effect of inactivating the endogenous pancreatic enzymes. The DMEM-F12 medium with pancreas tissue scrapings was added
30 to 5 ml of cold PBS.

The scraped tissue fragments were discarded and the remaining PBS (and DMEM-F12/FSA) (containing the stem cells)

was added to 10 ml of cold PBS and centrifuged at 1200 rpm for 5 minutes. The PBS (and DMEM-F12/FSA) was suctioned off and 10 ml of 0.05% trypsin was added to the sediment and incubated in a 37°C water bath for 5 minutes. The trypsin
5 was inactivated by adding a fresh 10 ml of DMEM-F12 and 10% FSA. The specimens were triturated with a Pasteur pipette until the tissue was in a fine suspension, then triturated again with a reduced bore Pasteur micropipette until the tissue was dissociated. The specimens were centrifuged at
10 1200 rpm for 5 minutes. The sediment was resuspended in complete DMEM/F12 supplemented with 20 ng/ml of EGF and bFGF and placed in an incubator at 37°C with 5% CO₂. All specimens, from each gland, contained many small, round neuroendocrine stem cells.

15 This same procedure can be used to extract and isolate neuroendocrine stem cells from other glands of the body, e.g., the hypothalamus, pituitary, parathyroid, and thyroid, and from along the intestinal tract and the aorta.

Other Embodiments

20 It is to be understood that while the invention has been described in conjunction with the detailed description thereof, that the foregoing description is intended to illustrate and not limit the scope of the invention, which is defined by the scope of the appended claims.

25 Other aspects, advantages, and modifications are within the scope of the following claims.